Original article

Cheating emergences in the arbuscular mycorrhizal mutualism: a network and phylogenetic analysis

Benoît Perez-Lamarque [1, 2], Marc-André Selosse [1, 3], Maarja Öpik [4], Hélène Morlon [2], Florent Martos [1]

[1] *Institut de Systématique, Évolution, Biodiversité (ISYEB), Muséum national d'histoire naturelle, CNRS, Sorbonne Université, EPHE, Université des Antilles; CP39, 57 rue Cuvier 75 005 Paris, France*

[2] *Institut de biologie de l'École normale supérieure (IBENS), École normale supérieure, CNRS, INSERM, PSL Université Paris, 46 rue d'Ulm, 75 005 Paris, France*

[3] *Department of Plant Taxonomy and Nature Conservation, University of Gdansk, Wita Stwosza 59, 80-308 Gdansk, Poland*

[4] *University of Tartu, 40 Lai Street, 51 005 Tartu, Estonia*

Corresponding author:
Benoît Perez-Lamarque, ENS, 46 rue d'Ulm, 75 005 Paris, France,
email: benoit.perez@ens.fr

Content: Words in the main text: 5,570, including introduction (1,088), materials and methods (1,959), results (934), and discussion (1,589).
The manuscript contains 2 tables and 4 color figures, and 8 supplementary tables and 6 supplementary figures.

Abstract

- While mutualisms are widespread and essential in ecosystem functioning, the emergence of uncooperative cheaters threatens their stability, unless there are functional or evolutionary mechanisms limiting cheaters interactions.
- Here, we evaluated the constraints upon mycoheterotrophic (MH) cheating plants in the mutualistic interaction network of autotrophic (AT) plants and arbuscular mycorrhizal fungi. For this purpose, we assembled a world-scale network of >25,000 interactions in order to investigate *(i)* the specialization and *(ii)* the phylogenetic distribution of MH or AT plants and their respective fungal partners.
- We show that MH cheating repeatedly evolved in the vascular flora, suggesting low phylogenetic constraint for plants. However, MH cheaters are significantly more specialized than AT plants, and their fungi also appear more specialized and more closely related than fungi of AT plants, which suggest that cheaters are specifically isolated into modules by functional constraints
- This unprecedented comparison of MH vs. AT plants thus reveals that MH cheating is most likely constrained by the specialization of phylogenetically conserved cheating-susceptible fungi, which suggests mechanisms for avoidance of these fungi. Beyond the mycorrhizal symbiosis, our approach highlights an empirical multiple-partners mutualistic system illustrating that the overall persistence of mutualism can be linked to functional constraints upon cheating emergences.

Keywords: arbuscular mycorrhiza, mutualism, cheaters, mycoheterotrophy, ecological networks, reciprocal specialization, phylogenetic constraint.

Introduction

Mutualistic interactions are ubiquitous in nature and largely contribute to generate and maintain biodiversity (Bronstein, 2015). Since benefits in mutualism often come at a cost for cooperators, some species called *cheaters* evolved an adaptive uncooperative strategy by retrieving benefits from an interaction without paying the associated cost (Sachs *et al.*, 2010). Although cheating compromises the evolutionary stability of mutualisms (Ferriere *et al.,* 2002), its evolutionary emergence is often limited by factors securing the persistence of mutualism (Bronstein *et al.*, 2003; Frederickson, 2013; Jones *et al.*, 2015). For instance, cooperators often favor the most rewarding partners (e.g. conditional investment; Roberts & Sherratt, 1998), stop interactions with cheaters (Pellmyr & Huth, 1994), or even sanction them (Kiers et al. 2003). Cheating emergence can thus be constrained through physiological or biochemical mechanisms of the interaction and its regulation, a type of functional mechanisms hereafter referred to as *functional constraints*. In addition, these functional constraints, and potentially others such as geographic constraints, can either be evolutionary conserved or not. If they are, there will be *phylogenetic constraints* on the emergence of cheaters, as some species will be evolutionary predisposed to cheating, or predisposed to be cheated upon, while others will not (Gómez *et al.* 2010).

The framework of bipartite interaction networks, combined with the phylogeny of partners, is useful for understanding the constraints limiting the emergence of cheaters in mutualisms. Analyses of bipartite networks have been extensively used to showcase the properties of mutualistic interactions (Bascompte *et al.*, 2003; Rezende *et al.*, 2007; Martos *et al.*, 2012), such as their level of specialization (number of partners), nestedness (do specialists establish asymmetric specialization with partners that are themselves generalists?), and modularity (existence of distinct sub-networks; Bascompte & Jordano 2014). These studies have shown that mutualistic networks are generally nested with specialists establishing asymmetric specialization with more generalist partners, contrary to antagonistic networks that tend to be modular, with partners establishing reciprocal specialization (Thebault & Fontaine, 2010). However, few analyses of bipartite networks include cheaters and analyze their specialization, nestedness and modularity (Fontaine *et al.*, 2011). Joffard *et al.* (2018) showed that specialization of orchids toward pollinators was higher in deceptive cheaters than in

cooperative nectar-producing species. Considering the overall effect of cheaters on network structure, Genini *et al.* (2010) showed that a network dominated by cooperative pollinators was nested, whereas another network dominated by nectar thieving insects was more modular. If cheaters specialize and form modules, this suggests that functional constraints limit the set of species that they can exploit. Additionally, if cheaters emerged only once on a phylogeny (*versus* repetitively), and/or if 'cheating-susceptible' partners are phylogenetically related (Merckx *et al.,* 2012), this suggests that cheating involves some rare evolutionary innovations (Pellmyr *et al,.* 1996) and/or that cheating-susceptibility is limited to few clades, meaning that cheating is phylogenetically constrained.

Here we evaluate functional and phylogenetic constraints upon cheating in the arbuscular mycorrhizal mutualism between plant roots and soil Glomeromycotina fungi (Selosse & Rousset, 2011; Jacquemyn & Merckx, 2019). This 400 Myr-old symbiosis concerns ca. 80% of extant land plants and several hundred fungal taxa (Davison *et al.,* 2015; van der Heijden *et al*., 2015). Arbuscular mycorrhizal fungi colonize plant roots and provide host plants with water and mineral nutrients, in return for carbohydrates. Although obligate for both partners, this symbiosis is generally diffuse and not very specific (van der Heijden *et al.*, 2015), since multiple fungi colonize most plants, while fungi are usually shared among surrounding plant species (Verbruggen *et al.* 2012). Thus, fungi interconnect plant individuals of different species, and allow resource movement between plants (Selosse *et al,.* 2006). This allowed the emergence of achlorophyllous cheating plants, called mycoheterotrophic (MH) plants, which obtain carbon from their mycorrhizal fungi that are themselves fed by surrounding autotrophic plants (AT) (Selosse & Rousset 2011; Merckx, 2013). Some of these plant species are entirely MH (*EMH*) over their lifecycle, while others are MH only at early stages before turning AT (initially MH, *IMH*), therefore shifting from cheater to potentially cooperative (Merckx, 2013). Unlike other cheating systems where cheating cost affects mostly direct partners (e.g. in plant pollination), MH cheaters involve not only a direct cost to their fungal partners but also a projected cost (i.e. a cost transmitted through the network) on the interconnected AT plants, whose photosynthesis supplies the carbon.

122 We evaluate functional constraints on MH by analyzing specialization, nestedness and
123 modularity in the plant mycorrhiza interaction network. MH plants are thought to be
124 specialists interacting with few fungal species (Leake, 1994; Merckx, 2013), but whether
125 or not these species are unusually specialized compared to AT plants is still debated
126 (Merckx *et al.*, 2012). MH plants could specialize on few fungal species if functional
127 constraints limit the set of fungi they can exploit, and evolve more efficient nutritional
128 stealing strategies (Blüthgen *et al.,* 2007). In terms of nestedness and modularity,
129 arbuscular mycorrhizal networks are generally nested (Chagnon *et al.*, 2012), but how
130 MH plants affect their structure has yet to be investigated. Because MH species indirectly
131 rely on AT plants for carbon nutrition, interacting with generalist fungi could provide
132 access to various carbon sources, and this would increase nestedness. Conversely, AT
133 plants could sanction interactions with fungi associated with MH plants because of the
134 projected cost; this would isolate cheating interactions within antagonistic modules and
135 increase modularity. Establishment of extreme reciprocal specialization between one
136 MH plant and one fungus seems unlikely though, since a AT carbon source is required.

137

138 With regards to phylogenetic constraints on MH, we already know that MH cheating
139 strategies evolved multiple times (Merckx, 2013), generating monophyletic groups of
140 MH plants, which suggests weak phylogenetic constraints on the emergence of cheating
141 in plants. However, it seems that fungi interacting with independent MH plant families
142 are phylogenetically close, suggesting that there are phylogenetic constraints on the
143 fungal side (Merckx *et al*., 2012). This needs to be confirmed in a larger phylogenetic
144 context including the fungi of AT plants. Indeed, if a set of phylogenetically close fungi
145 interact with all MH lineages, an important follow-up question is whether these fungi
146 were derived independently from AT ancestors, or whether they were acquired by
147 symbiont shift from other MH plants during new MH emergences.

148

149 We assess functional and phylogenetic constraints upon the emergence of MH cheaters
150 in the arbuscular mycorrhizal mutualism using a combination of network and
151 phylogenetic analyses. We analyze the largest available database on arbuscular
152 mycorrhizal fungi and their host plants (Öpik *et al.*, 2010), which we updated for this
153 analysis (see methods), and which includes both AT plants and most MH plant lineages.

154 ## Materials and Methods

155

156 ***Analytical framework***

157 Figure 1 summarizes the main analyses we carried to evaluate functional and
158 phylogenetic constraints upon the emergence of MH plants. We first assembled a
159 bipartite network representing which plants interact with which fungi. Next, we
160 analyzed functional constraints using tools from ecological networks (Fig. **1b**).
161 Functional constraints on MH cheaters (or MH-associated fungi) should be reflected by
162 the specialization of MH cheaters (or MH-associated fungi) towards few fungal (or plant)
163 partners. Strong constraints on both sides should drive reciprocal specialization and
164 isolation of MH cheaters and MH-associated fungi into modules, thus decreasing
165 nestedness. Finally, we analyzed phylogenetic constraints by analyzing the phylogenetic
166 distributions of MH cheaters and MH-associated fungi (Fig. **1a**).

167

168 ***MaarjAM database and interaction matrix***

169 MaarjAM database is a web-based database (http://maarjam.botany.ut.ee; accessed in
170 June 2019 after a very recent update) of publicly available sequences of
171 Glomeromycotina fungi, with information on the host plants, geographical location and
172 biomes for the recorded interactions (Öpik *et al.*, 2010, Davison *et al.*, 2015). We used an
173 approach with a compiled network, where all locally described physical mycelial
174 interactions between species are merged and studied at larger scales (as in Joffard *et al.*,
175 2018; Braga *et al.*, 2018). Although such a compiled network can be sensitive to several
176 biases (see Discussion), it is a unique opportunity to study MH cheating emergence in a
177 large evolutionary and ecological picture (e.g. Werner *et al.*, 2018). Among the 41,989
178 interactions between plants and Glomeromycotina, we filtered out the data from
179 MaarjAM for the fungi to satisfy the following criteria (Supporting Information Table
180 S1): *(i)* amplification of the 18S rRNA gene, *(ii)* fungus identified from plant roots (i.e.
181 excluding soil samples and anthropogenic sites), *(iii)* interaction in a natural ecosystem
182 (i.e. excluding anthropogenic or highly disturbed ecosystems), *(iv)* host plant identified
183 at the species level, and *(v)* a virtual taxon (VT) assignation available in MaarjAM. The
184 VTs are a classification (= species proxy) of arbuscular mycorrhizal fungi designed by
185 applying a ≥97% sequence similarity threshold to the 18S rRNA sequences, and by
186 running phylogenetic analysis to ensure VT monophyly (Öpik *et al.*, 2013, 2014). The

187 filtered dataset yielded a binary interaction matrix of 490 plant species (hereafter
188 'plants'), 351 VT (hereafter 'fungi'), and 26,350 interactions, resulting from the
189 compilations of 112 publications from worldwide ecosystems (Supporting Information
190 Fig. **S6**; Supporting Information Table **S1**). In order to estimate the sampling fraction of
191 Glomeromycotina fungi in our dataset, we performed rarefaction curves of the number
192 of fungal VTs as a function of the sampling fraction (on the observed number of
193 interactions or on the number of sampled plant species) and we estimated the total
194 number of units using the '*specpool*' function ('*vegan'* R-package, based on Chao index;
195 Oksanen *et al.*, 2019).

196

197 ***Phylogenetic reconstructions***

198 We aligned consensus sequences of the 351 fungi with the software MUSCLE (Edgar,
199 2004), and ran a Bayesian analysis using BEAST2 to reconstruct the fungal phylogeny
200 (Bouckaert *et al.* 2014, see Supplementary methods). We obtained the phylogenetic
201 relationships between the 490 host plants by pruning the time-calibrated supertree
202 from Zanne *et al.* (2013) using Phylomatic (http://phylodiversity.net/phylomatic/). We
203 also used the Open Tree of Life website (http://opentreeoflife.org) and the '*rotl*' package
204 (Michonneau et al. 2016) of R (R Core Team, 2019) for grafting 41 plant taxa missing in
205 the pruned supertree (as polytomies at the lowest taxonomy level possible, see
206 Supplementary methods). We set tree root calibrations at 505 million years (Myr) for
207 the fungi (Davison *et al.*, 2015) and 440 Myr for the plants (Zanne *et al.*, 2013).

208

209 ***Nature of the interaction***

210 We assigned to each plant its 'nature of the interaction' with fungi according to its
211 carbon nutrition mode: autotroph (AT, n=434, 88.6%), entirely mycoheterotroph (EMH,
212 n=41, 8.4%), or initially mycoheterotroph (IMH, n=15, 3.1%). According to an on-line
213 database (http://mhp.myspecies.info/category/myco-heterotrophic-plants/) and
214 individual publications (Boullard, 1979; Winther & Friedman, 2008; Field et al. 2015),
215 EMH species belong to six monophyletic families [Burmanniaceae (25 spp.),
216 Gentianaceae (6 spp.), Triuridaceae (4 spp.), Polygalaceae (4 spp.), Corsiaceae (1 sp.),
217 and Petrosaviaceae (1 sp.)], and IMH species belong to three families [Ophioglossaceae
218 (ferns; 5 spp.), Psilotaceae (ferns; 2 spp.), and Lycopodiaceae (clubmoss; 8 spp.)]. We

219 assigned each fungus to three categories: 'AT-associated' if the fungus interacts with AT
220 plants only (n=280, 79.8%), 'EMH-associated' if the fungus interacts with at least one
221 EMH plant (n=54, 15.4%), 'IMH-associated' if the fungus interacts with at least one IMH
222 plant (n=23, 6.6%), or 'MH-associated' if the fungus interacts with at least one EMH or
223 IMH plant (n=71, 20.2%; Supporting Information Table **S2**). Only five MH-associated
224 fungi are both EMH- and IMH-associated. Although our dataset unfortunately does not
225 include all MH plants (it includes 18 publications), it covers a fair representative range
226 of the mycoheterotrophs in the arbuscular mycorrhizal symbiosis (Jacquemyn & Merckx,
227 2019).

228

229 ***Network nestedness and specialization of cheaters***

230 In order to assess functional constraints, we tested the effect of MH cheating on network
231 structure. First, we measured nestedness in: *(i)* the overall network (490 plants, 351
232 fungi, and 26,350 interactions), *(ii)* the network restricted to AT plants only (434, 344,
233 and 26,087) and *(iii)* the network formed by EMH and IMH plants only (56, 71, and 263),
234 using the function '*NODF2*' in the R package '*bipartite'* (Dormann et al. 2008). Positive
235 NODF values (nestedness metric based on overlap and decreasing fill; see *List of*
236 *abbreviations* in Supplementary material) indicate nested networks. We tested the
237 significance of nestedness values by using two types of null models (*N*= 100 for each
238 type): both maintain the connectance (proportion of observed interactions) of the
239 network, but the first model (*'r2dtable'* from *'stats'* R package - *null model 3*) also
240 maintains the marginal sums (the sums of each row and each column) while the less
241 stringent second model (*'vaznull'* from '*bipartite'* R package - *null model 2*) produces
242 slightly different marginal sums (interactions are randomized with species marginal
243 sums as weights, and each species must have at least one interaction). We calculated the
244 Z-score, which is the standard deviation between the observed value and the mean of
245 the distribution of null-model networks (Z-scores greater than 1.96 validates a
246 significant nestedness with an alpha risk of 5%).

247 Second, to further evaluate the specialization of MH cheaters, we computed several
248 network indices for each plant. The degree (*k*) is the number of partners with which a
249 given plant or fungus interacts in the bipartite network. The degree is high (resp. low)
250 when the species is generalist (resp. specialist). The partner specialization (*Psp*) is the

251 mean degree (*k*) averaged for all the fungal partners for a given plant species (Taudiere
252 *et al.*, 2015): a high (resp. low) *Psp* characterizes a species interacting mainly with
253 generalist (resp. specialist) partners. Simultaneously low *k* and *Psp* values feature a
254 reciprocal specialization. Then, in order to detect specialization at clade scale toward
255 partners, for any given clade of every node in the plant or fungus phylogenies, we
256 calculated the partner fidelity (*Fx*) as the ratio of partners exclusively interacting with
257 this particular clade divided by the total number of partners interacting with it. We
258 consider the clade as 'faithful' and the corresponding set of partners as 'clade-specific'
259 when *Fx*>0.5 (i.e., more than 50% exclusive partners). We tested whether *k* and *Psp*
260 were statistically different among AT, EMH and IMH plants using non-parametric
261 Kruskal-Wallis tests and pairwise Whitney U tests. We also replicated the statistical tests
262 without the IMH Lycopodiaceae, because of their different network patterns (see
263 Results). We used an analysis of covariance (ANCOVA) to test the effect of the nature of
264 the interaction on partner fidelity *Fx* accounting for clade size, which corrects the bias of
265 having high partner fidelity *Fx* in older clades including many plants.

266 To assess the significance of *k* and *Psp* values, we built null-model networks (*N*= 1,000)
267 using the function '*permatfull*' in the '*vegan*' R package *(null model 1)*, keeping the
268 connectance constant but allowing different marginal sums.

269 Third, we used projected networks to detect cases of extreme reciprocal specializations
270 leading to independent modules. The projected network is the unipartite plant-plant
271 network derived from the bipartite plant-fungus network, where plants interact
272 together if they share at least one fungus. We computed the projected degree, that is the
273 degree of plants in the unipartite network.

274 To confirm that the patterns of specialization at the global scale hold at a more local
275 scale, we reproduced the analyses of specialization (*k* and *Psp*) in two continental
276 networks in South America and Africa since they represent a high number of
277 interactions and of MH species (Supporting Information Fig. **S5**).

278

279 ***Phylogenetic distribution of cheating***

280 In order to assess phylogenetic constraints, we explored the phylogenetic distribution of
281 MH cheaters and MH-associated fungi. First, we investigated the phylogenetic

282 distribution of mycoheterotrophy, i.e. if MH plants and MH-associated fungi are more or
283 less phylogenetically related than expected by chance (i.e. patterns of clustering *versus*
284 overdispersion). We computed the net relatedness index (NRI) and the nearest taxon
285 index (NTI) using the '*PICANTE*' R package (Kembel *et al.*, 2010). NRI quantifies the
286 phylogenetic structure of a species set based on the mean pairwise distances, whereas
287 NTI quantifies the terminal structure of the species set by computing the mean
288 phylogenetic distance to the nearest taxon of every species (Gotelli & Rohde, 2002). To
289 standardize the indices, we generated 999 null models for each of the following option
290 '*taxa.labels*' (shuffles the taxa labels). Significant positive (resp. negative) NRI and NTI
291 values indicate phylogenetic clustering (resp. overdispersion). We computed these
292 indices *(i)* on the plant phylogeny to evaluate the phylogenetic structure of EMH and
293 IMH plants distribution, and *(ii)* on the fungal phylogeny to investigate if MH-associated
294 fungi are phylogenetic structured (we successively tested the distribution of MH-, EMH-,
295 and IMH-associated fungi, and then of the fungi associated with each specific MH family).
296 Similarly, for each plant, we computed the partners' mean phylogenetic pairwise
297 distance (*MPD*) that is the average phylogenetic distance across pairs of fungal partners
298 (Kembel *et al.*, 2010): a low value of *MPD* indicates that the set of partners is constituted
299 of closely related species. The effect of MH cheating on *MPD* values and its significance
300 was evaluated as for *k* and *Psp* values above.

301 Second, in order to assess whether fungal partners of a given MH family were derived
302 from fungal partners of AT ancestors or secondary acquired from other MH lineages, we
303 compared in an evolutionary framework the sets of fungi associated with plants with
304 different natures of the interaction. To do so, we computed the unweighted Unifrac
305 distance (Lozupone & Knight, 2005) between sets of fungi interacting with each pair of
306 plants in the network. For each one of the seven MH families, we compared the Unifrac
307 distances across *(i)* every pairs of plant species of this family, *(ii)* every pairs made of
308 one plant of this family and one plant of the most closely related AT family (see Table **2**
309 for AT families), *(iii)* every pairs made of one plant of this family and one plant belonging
310 to other MH families, and *(iv)* every pairs made of one plant of this family and one more
311 distant AT plant (i.e. all AT plants except those of the most closely related AT family).
312 This analysis was not performed on MH Petrosaviaceae, represented by only one species
313 and too divergent to define a reliable AT sister clade.

314 We tested differences between groups of distances using Mann-Whitney U tests. We also
315 performed a principal coordinates analysis (PCoA) from all the UniFrac dissimilarities of
316 sets of fungal partners, and tested the effect of the nature of the interaction on the two
317 principal coordinates, using Kruskal-Wallis tests. Finally, to examine the extent to which
318 the nature of the interaction predicts fungal partners, we used permutational analysis of
319 variance (PerMANOVA, '*adonis*' function in R package '*vegan*'), with 10,000
320 permutations.
321

322 ## Results

323 We analyzed the global arbuscular mycorrhizal network filtered from the MaarjAM
324 database (490 plants, 351 fungi, and 26,350 interactions) combined with the
325 reconstructed phylogenetic trees of both partners (Fig. **2**). We estimated a total number
326 of 373 +/- 9 fungi (Chao index), which indicates that the 351 fungi in the dataset
327 included most of the arbuscular mycorrhizal fungal diversity (94% +/- 2%; Supporting
328 Information Fig. S1).

329

330 ***Network nestedness and specialization of cheaters***

331 The overall network had a significant positive nestedness value (Z-score=9.2, $P=1.10^{-20}$,
332 Supporting Information Table **S4**). Nestedness increased when considering only AT
333 plants (Z-score=16.6, $P=8.10^{-62}$), whereas it was not significant in the network of only
334 MH plants (Z-score=1.44, P=0.075): MH plants reduced nestedness, signifying that they
335 display higher reciprocal specializations. In addition to a main module encompassing
336 most species (481 out of 490 plants and 346 out of 351 fungi), we found three small
337 independent modules (Supporting Information Table S3): *(i)* 6 IMH Lycopodiaceae
338 plants and 3 exclusive fungi (*Glomus* VT127, VT158, VT394); *(ii)* two AT plants from salt
339 marshes (*Salicornia europaea* and *Limonium vulgare*) with one *Glomus* (VT296); and *(iii)*
340 the EMH *Kupea martinetugei* with a unique *Glomus* (VT204).

341 From the degrees (*k*), we found that EMH and IMH plants are significantly more
342 specialized than AT plants by interacting with on average more than five time less fungi
343 ($P=1.4.10^{-17}$, Kruskal-Wallis test; Fig. **3a**, Table **1**). Partner specializations (*Psp*)
344 indicated that MH plants interact with more specialized fungi (MH-associated fungi
345 themselves interact on average with two times fewer plants; $P=5.6.10^{-11}$; Fig. **3a**). We
346 found similar evidence for MH reciprocal specializations by reanalyzing the network
347 excluding the family Lycopodiaceae (Table **1**; significance assessments using null models
348 are in Supporting Information Table **S5**). This pattern of reciprocal specialization of MH
349 plants and their associated fungi held at a smaller geographical scale in the African and
350 South American networks (Supporting Information Fig. **S5**; Supporting Information
351 Table **S8**; yet the difference was not significant for *Psp* in the South American network,
352 probably due to the small number of species and the low power of the statistical tests).

353 The partner fidelity index (*Fx*) showed that very few plant and fungi clades interact with
354 'clade-specific' partners (i.e. *Fx*>0.5), and most fungi are shared between different plant
355 clades (Fig. **3c**). Among exceptions, however, the clade of IMH Lycopodiaceae was
356 characterized by a high partner fidelity index (*Fx*>0.8), reflecting a strong association
357 with a clade of 3 Lycopodiaceae-associated fungi (Supporting Information Fig. **S4**). Thus,
358 not only these 6 Lycopodiaceae species and their fungal partners form an independent
359 module, but also the Lycopodiaceae-associated fungi form a monophyletic clade within
360 Glomeromycotina. The estimated clade age was 250 Myr for the Lycopodiaceae and 49
361 Myr for the Lycopodiaceae-associated fungi (Fig. **3d**), which diverged 78 Myr ago from
362 the other *Glomus* fungi.

363

364 ***Phylogenetic distribution of cheating***

365 The partners' mean phylogenetic pairwise distance (*MPD*) indicated that both EMH- and
366 IMH-associated fungi (or even all MH-associated fungi) are phylogenetically more
367 closely related than AT-associated fungi ($P=1.6.10^{-6}$; Table **1**; Fig. **3b**). NRI and NTI
368 values (Supporting Information Table **S7**) also confirmed a significant clustering of MH-,
369 EMH- and IMH-associated fungi on the fungal phylogeny; this clustering held at the
370 family level for fungi associated with each of four main MH families (namely
371 Burmanniaceae, Triuridaceae, Polygalaceae, and Ophioglossaceae). On the plants side,
372 only the EMH plants were significantly clustered, mainly because they all are
373 angiosperms and mostly monocots, but this does not apply for MH plants in general, nor
374 for IMH plants (Supporting Information Table **S7**). These phylogenetic clusters are
375 visually noticeable on fungal and plant phylogenetic trees (Supporting Information Fig.
376 **S2** and **S3**). This demonstrates that although MH cheating evolved several times
377 independently in plants, MH plants interact mainly with closely related fungi (see also
378 Fig. **2**).

379 Looking specifically at the fungi shared among EMH and IMH plants highlighted
380 differences between EMH and IMH plants (Table **2**). While the IMH Lycopodiaceae
381 family forms an independent module with three specific *Glomus* VTs, another IMH family
382 Ophioglossaceae also had 2 exclusive fungi (*Glomus* VT134 and VT173) among a total of
383 15 fungi. When comparing the fungi shared between MH families (Table **2**), mainly two

384 closely related families, Burmanniaceae and Triuridaceae, tend to share some fungi with
385 other MH families.

386 The decomposition of Unifrac dissimilarities between sets of fungal partners using a
387 principal coordinate analysis, showed a clear pattern of clustering of MH species,
388 supporting that the set of fungal partners associated with MH cheaters are more similar
389 than expected by chance ($P<1.\ 10^{-16}$ for PCoA1; $P= 9.10^{-3}$ for PCoA2; Fig. **4a**). Similarly,
390 the PerMANOVA analysis indicated that the nature of the interaction (IMH, EMH, or AT)
391 predicts 6.5% of the variance (P=0.0001). By comparing the Unifrac dissimilarities
392 between sets of fungal partners according to the nature of the interaction and plant
393 family relatedness, we observed that all MH families have fungal partners more similar
394 to each other than those of other AT families (Fig. **4b**; Supporting Information Table **S6**).
395 Some families (Burmanniaceae, Polygalaceae, Triuridaceae, Lycopodiaceae, and
396 Ophioglossaceae) had fungal partners significantly more similar to partners interacting
397 with their closest AT relatives ($P>0.05$) than to partners interacting with other AT
398 families ($P<10^{-16}$). This supports a phylogenetic conservatism of fungal partners during
399 the evolution of MH nutrition in these families. For other MH families (Corsiaceae,
400 Gentianaceae, and Psilotaceae), fungal partners were significantly more similar to
401 partners interacting with other MH families than to partners interacting with their
402 closest AT relatives, the latter being as distant as other AT families (Supporting
403 Information Table **S6**). This supports a shift to new fungal partners correlated with the
404 evolution of MH nutrition in these three families.

405

406 ## Discussion

407

408 We assessed constraints upon the emergence of MH cheating in the mutualistic
409 arbuscular mycorrhizal network. Although this network was nested, we found evidence
410 for reciprocal specialization in the case of MH cheaters (specialists) and their fungal
411 partners (also specialists). We even observed unexpected, extreme reciprocal
412 specialization for some IMH families having exclusive fungi. Finally, we found that
413 independently emerged MH families share many closely related fungi, and that in some
414 of these families fungal partners were derived from AT ancestors, while in others they
415 were acquired by symbiont shift, suggesting different evolutionary pathways leading to
416 MH.

417

418 ***Cheaters are isolated by reciprocal specialization***

419 We confirmed that MH plants are more specialized toward few mycorrhizal partners
420 than AT plants (Merckx *et al.*, 2012), and showed for the first time that their fungal
421 partners are more specialists than fungi associated with AT plants. This reciprocal
422 specialization is not strict (with the exception of Lycopodiaceae, see below), since MH
423 cheaters and their fungal partners need some connection to AT plants for carbon
424 providing. These findings contrast with the general prediction that, in stable and
425 resilient mutualistic networks, specialists should interact with generalist partners
426 (asymmetrical specialization), resulting in nested networks. We indeed find that MH
427 cheaters lower nestedness in the arbuscular mycorrhizal network. The observed trend
428 toward reciprocal specialization suggests that cheating is an unstable ecological and
429 evolutionary strategy, which could explain the relatively recent origin of MH clades (Fig.
430 **2**). Whatever its origin, the reciprocal specialization of cheaters and their partners was
431 also suggested in other mutualisms (Genini *et al.*, 2010). A parasitic nature of EMH
432 plants has often been guessed (Bidartondo, 2005; Merckx, 2013) but without direct
433 support, in the absence of data on fitness of fungal partners and AT plants linked to MH
434 plants (van der Heijden *et al.*, 2015). Our analysis *a posteriori* supports the view of EMH
435 plants as parasitic cheaters.

436

437 There are several non-exclusive explanations to this reciprocal MH specialization. First,
438 functional constraints may act since conditional investment and partner choice occur in

439 the mycorrhizal symbiosis (Kiers *et al.,* 2011): each partner rewards and interacts
440 specifically with the most mutualistic of the many partners they encounter in soil. MH
441 cheaters were able to successfully avoid these constraints with only few specific MH-
442 susceptible fungi with which they now faithfully interact through a parasitic
443 specialization (Selosse & Rousset, 2011). On the fungal side, we can speculate that
444 'cheated' MH-susceptible fungi that provide MH plants with carbon have greater carbon
445 costs for AT plants than other fungi, and that AT plants therefore avoid interactions with
446 these fungi. This would result in reciprocal specialization, and the partial isolation of MH
447 cheaters and their fungal partners from the mutualistic network. The fact that MH plants
448 tend to occur in local patches of low fertility (Gomes *et al.*, 2019) could suggest that in
449 such habitats where access to essential mineral nutrients is limiting, it is even
450 advantageous for AT plants to interact with poorly rewarding fungal partners such as
451 those associated with MH plants.
452 Second, if it exists a negative trade-off between the cooperativeness of a fungus and its
453 susceptibility to MH plants (for unknown physiological reason, e.g. linked to
454 mechanisms of carbon exchange), cheating MH plants would mainly interact with poorly
455 cooperative fungi, which may be therefore intrinsically avoided by autotrophs.
456
457 An in-depth sampling of mycorrhizal interactions and partners' abundances in various
458 MH local communities would be required to confirm that reciprocal specialization
459 indeed occurs in local communities. Indeed, we observed reciprocal specialization in a
460 large-scale interaction network compiled from mycorrhizal interactions described in
461 different ecosystems around the world, not in locally described physical mycelial
462 networks. This allowed analyzing a global ecological pattern, representing the complete
463 evolutionary history of the partners, and is justified by the very low endemism of
464 arbuscular mycorrhizal fungi and thus the absence of strong geographic structure
465 (Davison *et al.*, 2015; Savary *et al.*, 2018). It is noteworthy that similar patterns of
466 specialization were found in the African and South American networks during this study
467 (Supporting Information Fig. **S5**). On the other hand, a species may appear relatively
468 more specialist in a global network than it actually is in local communities. Reciprocal
469 specialization at the local scale would still need to be confirmed.
470

471 ***Independent emergences of EMH cheating converge on closely-related susceptible***
472 ***fungi***
473 MH cheating emerged multiple times at different positions of the phylogeny of vascular
474 land plants, indicating weak phylogenetic constraints. This likely results from the low
475 specificity in the arbuscular mycorrhizal symbiosis allowing convergent interactions
476 (Bittleston *et al.*, 2016) in different plant clades. This would have happened during the
477 evolution of MH cheaters with similar MH-susceptible fungi. Thus, functional constraints
478 appear as the main barrier to cheating emergences in the arbuscular mycorrhizal
479 mutualism.
480
481 There are however phylogenetic constraints on the fungal side. We found only few
482 fungal clades interacting with independent MH lineages, and they were phylogenetically
483 related in this study as in (Merckx *et al.*, 2012). The susceptibility of fungi to MH
484 cheaters is difficult to discuss, because we know little about physiological and symbiotic
485 traits of arbuscular mycorrhizal fungi (Chagnon et al. 2013; van der Heijden & Scheublin
486 2007). Yet, interactions between MH cheaters and MH-susceptible fungi likely represent
487 exceptions to the partners choice, which is generally considered to constitutively
488 maintain the stability of this mutualism (Selosse & Rousset, 2011).
489
490 The acquisition of MH-susceptible fungi depends on the MH family. In MH families such
491 as Burmanniaceae, fungal partners are closely-related to the fungal partners of AT
492 relatives, suggesting that the MH-associated fungi are derived from the fungal partners
493 of cooperative AT ancestors. In other MH families, such as Gentianaceae or Corsiaceae,
494 fungal partners are more closely-related to fungal partners of other MH families than to
495 AT relatives, suggesting that the MH-associated fungi were secondarily acquired rather
496 than derived from the partners of AT ancestors. A few MH lineages lack closest AT
497 relatives in our analysis (e.g. MH Gentianaceae should be compared to AT Gentianaceae,
498 which are not represented in the MaarjAM database), which may bias our analyses
499 towards supporting secondary transfer from other MH plants rather than acquisition
500 from AT ancestors. Still, similar fungi were found in MH Burmanniaceae and their
501 closest AT relative after a 110 Myrs-old divergence, while MH Gentianaceae and their
502 closest AT relative have distinct fungal partners after a divergence of only 52 Myrs.
503

504 Interestingly, all EMH families are evolutionarily relatively recent: the oldest monocot
505 EMH families, such as Burmanniaceae and Triuridaceae, are only 110-130 Myr old, and
506 the dicot EMH families Gentianaceae and Polygalaceae are even more recent (around 50-
507 60 Myr; Zanne *et al.*, 2013; Fig. **2**; Table **2**). The oldest MH families show conservatism
508 for fungal partners while the most recently evolved ones display secondary acquisition.
509 We can speculate that mycoheterotrophy initially emerged in the monocot clade thanks
510 to suitable cheating-susceptible fungal partners; more recently evolved EMH plant
511 families (especially the dicots) then convergently reutilized these fungal partners.
512 Complementary analyses including more sampling of the MH families and their closest
513 AT relatives would be needed to test this speculation.
514
515 ***Independent networks and parental nurture in IMH***
516 Our results serendipitously revealed that two IMH families, Ophioglossaceae and
517 Lycopodiaceae, have exclusive mycorrhizal associations: they interact with fungi that do
518 not interact with any other plant family. In these families, the fungi are present during
519 both MH underground spore germination and in roots of adult AT individuals (Winther
520 & Friedman, 2007, 2008). AT adults likely act as the carbon source (Field *et al.*, 2015),
521 part of which is dedicated to the offspring. This further supports Leake *et al.* (2008)'s
522 hypothesis of parental nurture where germinating spores would be indirectly nourished
523 by surrounding conspecific sporophytes. Parental nurture is not universal to all IMH
524 families though; in the IMH Psilotaceae for example, fungal partners are shared with
525 surrounding AT plants (Winther & Friedman, 2009). In IMH independent networks, the
526 overall outcome for the fungus over the plant lifespan may actually be positive: fungi
527 invest in MH germinations that represent future carbon sources (Field *et al.*, 2015). In
528 other words, IMH plants do not cheat their exclusive fungi, but postpone reward. We
529 note however that the existence of independent networks for these families should be
530 confirmed in studies of local communities.
531
532 We found an extreme reciprocal specialization between Lycopodiaceae and a single
533 *Glomus* clade. Contrary to other early-diverging plant clades that tend to interact with
534 early-diverging fungal clades, the Lycopodiaceae (250-Myr-old) associate with a 49-My-
535 old clade that diverged 78 Myr ago from all other *Glomus* (Rimington *et al.,* 2018). Thus,
536 this highly specific interaction results from a secondary acquisition: some species of

Lycopodiaceae may have initially developed IMH with a wider set of fungi, and later evolved into a specific mutualistic parental nurture with their exclusive fungi, raising the possibility of a co-evolution between both clades.

Conclusions

Our analysis of MH cheating in the arbuscular mycorrhizal symbiosis highlights the role of functional constraints in preventing the emergence of cheaters in mutualisms. Partner choices or sanctions lead to reciprocal specialization which breaks the nested network into modules and isolates MH cheaters and their 'cheating-susceptible' partners. Phylogenetic constraints occur on the fungal but not the plant side, with independently emerged MH cheaters that convergently interact with closely related fungi. In addition, our results challenge the general cheater status of mycoheterotrophy, highlighting a dichotomy between true MH cheaters and possibly cooperative, IMH systems with parental nurture. Beyond the mycorrhizal symbiosis, we invite to use our combination of network and phylogenetic approaches to evaluate the strength of constraints upon cheating in other multiple-partner mutualisms (e.g. pollination or seed dispersal).

Acknowledgments

The authors thank members of the INEVEF team at ISYEB and BIODIV team at IBENS for helpful comments on the article. They also thank C. Fontaine for helpful discussions on network analyses.

This work was supported by a doctoral fellowship from the École Normale Supérieure de Paris attributed to BPL and the École Doctorale FIRE - Programme Bettencourt. MÖ was supported by the European Regional Development Fund (Centre of Excellence EcolChange) and the Estonian Research Council (IUT20-28). HM acknowledges support from the European Research council (grant CoG-PANDA).

Author contributions

All authors designed the study. MÖ gathered the data, BPL performed the analyses and wrote the first draft of the manuscript, and all authors contributed substantially to the writing and revisions.

Data Availability Statement

All the data used in this work are available in the MaarjAM database (https://maarjam.botany.ut.ee; Öpik *et al.*, 2010).

References

Bascompte J, Jordano P. 2014. *Mutualistic Networks* (Princeton University Press, Ed.). Princeton University Press.

Bascompte J, Jordano P, Melián CJ, Olesen JM. 2003. The nested assembly of plant-animal mutualistic networks. *Proceedings of the National Academy of Sciences of the United States of America* **100**: 9383–7.

Bidartondo MI. 2005. The evolutionary ecology of myco-heterotrophy. *New Phytologist* **167**: 335–352.

Bittleston LS, Pierce NE, Ellison AM, Pringle A. 2016. Convergence in Multispecies Interactions. *Trends in Ecology and Evolution* **31**: 269–280.

Blüthgen N, Menzel F, Hovestadt T, Fiala B, Blüthgen N. 2007. Specialization, Constraints, and Conflicting Interests in Mutualistic Networks. *Current Biology* **17**: 341–346.

Bouckaert R, Heled J, Kühnert D, Vaughan T, Wu C-H, Xie D, Suchard MA, Rambaut A, Drummond AJ. 2014. BEAST 2: a software platform for Bayesian evolutionary analysis (A Prlic, Ed.). *PLoS Computational Biology* **10**: e1003537.

Boullard B. 1979. Considérations sur la symbiose fongique chez les Ptéridophytes. *Syllogeus* **19 : 1-58**.

Braga MP, Guimarães PR, Wheat CW, Nylin S, Janz N. 2018. Unifying host-associated diversification processes using butterfly–plant networks. *Nature Communications* **9**: 5155.

Bronstein JL. 2015. *Mutualism* (JL Bronstein, Ed.). Oxford University Press.

Bronstein JL, Wilson WG, Morris WF. 2003. Ecological dynamics of mutualist/antagonist communities. *The American naturalist* **162**: S24-39.

Chagnon P-L, Bradley RL, Klironomos JN. 2012. Using ecological network theory to evaluate the causes and consequences of arbuscular mycorrhizal community structure. *New Phytologist* **194**: 307–312.

601 **Chagnon P-L, Bradley RL, Maherali H, Klironomos JN. 2013**. A trait-based framework
602 to understand life history of mycorrhizal fungi. *Trends in Plant Science* **18**: 484–491.
603 **Davison J, Moora M, Öpik M, Adholeya A, Ainsaar L, Bâ A, Burla S, Diedhiou AG,**
604 **Hiiesalu I, Jairus T, *et al.* 2015**. Global assessment of arbuscular mycorrhizal fungus
605 diversity reveals very low endemism. *Science (New York, N.Y.)* **349**: 970–3.
606 **Dormann CF, Gruber B, Fründ J. 2008**. Introducing the bipartite package: analysing
607 ecological networks. *R News* **8**: 8–11.
608 **Edgar RC. 2004**. MUSCLE: multiple sequence alignment with high accuracy and high
609 throughput. *Nucleic Acids Research* **32**: 1792–1797.
610 **Ferriere R, Bronstein JL, Rinaldi S, Law R, Gauduchon M. 2002**. Cheating and the
611 evolutionary stability of mutualisms. *Proceedings. Biological sciences* **269**: 773–80.
612 **Field KJ, Leake JR, Tille S, Allinson KE, Rimington WR, Bidartondo MI, Beerling DJ,**
613 **Cameron DD. 2015**. From mycoheterotrophy to mutualism: Mycorrhizal specificity and
614 functioning in Ophioglossum vulgatum sporophytes. *New Phytologist* **205**: 1492–1502.
615 **Fontaine C, Guimarães PR, Kéfi S, Loeuille N, Memmott J, van der Putten WH, van**
616 **Veen FJF, Thébault E. 2011**. The ecological and evolutionary implications of merging
617 different types of networks. *Ecology Letters* **14**: 1170–1181.
618 **Frederickson ME. 2013**. Rethinking mutualism stability: cheaters and the evolution of
619 sanctions. *The Quarterly review of biology* **88**: 269–95.
620 **Genini J, Morellato LPC, Guimarães PR, Olesen JM. 2010**. Cheaters in mutualism
621 networks. *Biology letters* **6**: 494–7.
622 **Gomes SIF, Bodegom PM, Merckx VSFT, Soudzilovskaia N. 2019**. Environmental
623 drivers for cheaters of arbuscular mycorrhizal symbiosis in tropical rainforests. *New*
624 *Phytologist*: nph.15876.
625 **Gómez JM, Verdú M, Perfectti F. 2010**. Ecological interactions are evolutionarily
626 conserved across the entire tree of life. *Nature* **465**: 918–921.
627 **Gotelli NJ, Rohde K. 2002**. Co-occurrence of ectoparasites of marine fishes: a null model
628 analysis. *Ecology Letters* **5**: 86–94.
629 **van der Heijden MGA, Martin FM, Selosse M-A, Sanders IR. 2015**. Mycorrhizal
630 ecology and evolution: the past, the present, and the future. *New Phytologist* **205**: 1406–
631 1423.
632 **van der Heijden MGA, Scheublin TR. 2007**. Functional traits in mycorrhizal ecology:
633 their use for predicting the impact of arbuscular mycorrhizal fungal communities on

634 plant growth and ecosystem functioning. *New Phytologist* **174**: 244–250.
635 **Jacquemyn H, Merckx VSFT**. **2019**. Mycorrhizal symbioses and the evolution of trophic
636 modes in plants (R Shefferson, Ed.). *Journal of Ecology*: 1365-2745.13165.
637 **Joffard N, Massol F, Grenié M, Montgelard C, Schatz B**. **2018**. Effect of pollination
638 strategy, phylogeny and distribution on pollination niches of Euro-Mediterranean
639 orchids (I Bartomeus, Ed.). *Journal of Ecology*.
640 **Jones EI, Afkhami ME, Akçay E, Bronstein JL, Bshary R, Frederickson ME, Heath KD,**
641 **Hoeksema JD, Ness JH, Pankey MS, *et al.* 2015**. Cheaters must prosper: reconciling
642 theoretical and empirical perspectives on cheating in mutualism (N van Dam, Ed.).
643 *Ecology Letters* **18**: 1270–1284.
644 **Kembel SW, Cowan PD, Helmus MR, Cornwell WK, Morlon H, Ackerly DD,**
645 **Blomberg SP, Webb CO**. **2010**. Picante: R tools for integrating phylogenies and ecology.
646 *Bioinformatics* **26**: 1463–1464.
647 **Kiers ET, Duhamel M, Beesetty Y, Mensah JA, Franken O, Verbruggen E, Fellbaum**
648 **CR, Kowalchuk GA, Hart MM, Bago A, *et al.* 2011**. Reciprocal rewards stabilize
649 cooperation in the mycorrhizal symbiosis. *Science* **333**: 880–882.
650 **Kiers ET, Rousseau RA, West SA, Denison RF**. **2003**. Host sanctions and the legume–
651 rhizobium mutualism. *Nature* **425**: 78–81.
652 **Leake JR**. **1994**. The biology of myco-heterotrophic ('saprophytic') plants. *New*
653 *Phytologist* **127**: 171–216.
654 **Leake JR, Cameron DD, Beerling DJ**. **2008**. Fungal fidelity in the myco-heterotroph-to-
655 autotroph life cycle of Lycopodiaceae: a case of parental nurture? *New Phytologist* **177**:
656 572–576.
657 **Lozupone C, Knight R**. **2005**. UniFrac: a new phylogenetic method for comparing
658 microbial communities. *Applied and environmental microbiology* **71**: 8228–35.
659 **Martos F, Munoz F, Pailler T, Kottle I, Gonneau C, Selosse M-A**. **2012**. The role of
660 epiphytism in architecture and evolutionary constraint within mycorrhizal networks of
661 tropical orchids. *Molecular Ecology* **21**: 5098–5109.
662 **Merckx VSFT**. **2013**. Mycoheterotrophy: An Introduction. In: Mycoheterotrophy. New
663 York, NY: Springer New York, 1–17.
664 **Merckx VSFT, Janssens SB, Hynson NA, Specht CD, Bruns TD, Smets EF**. **2012**.
665 Mycoheterotrophic interactions are not limited to a narrow phylogenetic range of
666 arbuscular mycorrhizal fungi. *Molecular Ecology* **21**: 1524–1532.

Michonneau F, Brown JW, Winter DJ. 2016. rotl : an R package to interact with the Open Tree of Life data (R Fitzjohn, Ed.). *Methods in Ecology and Evolution* **7**: 1476–1481.

Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, Mcglinn D, Minchin PR, O'hara RB, Simpson GL, Solymos P, *et al.* 2019. *vegan: Community Ecology Package, R package version 2.5-5*.

Öpik M, Davison J, Moora M, Zobel M. 2014. DNA-based detection and identification of Glomeromycota: the virtual taxonomy of environmental sequences. *Botany* **92**: 135–147.

Öpik M, Vanatoa A, Vanatoa E, Moora M, Davison J, Kalwij JM, Reier Ü, Zobel M. 2010. The online database MaarjAM reveals global and ecosystemic distribution patterns in arbuscular mycorrhizal fungi (Glomeromycota). *New Phytologist* **188**: 223–241.

Öpik M, Zobel M, Cantero JJ, Davison J, Facelli JM, Hiiesalu I, Jairus T, Kalwij JM, Koorem K, Leal ME, *et al.* 2013. Global sampling of plant roots expands the described molecular diversity of arbuscular mycorrhizal fungi. *Mycorrhiza* **23**: 411–430.

Pellmyr O, Huth CJ. 1994. Evolutionary stability of mutualism between yuccas and yucca moths. *Nature* **372**: 257–260.

Pellmyr O, Leebens-Mack J, Huth CJ. 1996. Non-mutualistic yucca moths and their evolutionary consequences. *Nature* **380**: 155–156.

R Core Team. 2019. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Revell LJ. 2012. phytools: An R package for phylogenetic comparative biology (and other things). *Methods in Ecology and Evolution* **3**: 217–223.

Rezende EL, Lavabre JE, Guimarães PR, Jordano P, Bascompte J. 2007. Non-random coextinctions in phylogenetically structured mutualistic networks. *Nature* **448**: 925–928.

Rimington WR, Pressel S, Duckett JG, Field KJ, Read DJ, Bidartondo MI. 2018. Ancient plants with ancient fungi: liverworts associate with early-diverging arbuscular mycorrhizal fungi. *Proceedings of the Royal Society B: Biological Sciences* **285**: 20181600.

Roberts G, Sherratt TN. 1998. Development of cooperative relationships through increasing investment. *Nature* **394**: 175–179.

Sachs JL, Russell JE, Lii YE, Black KC, Lopez G, Patil AS. 2010. Host control over infection and proliferation of a cheater symbiont. *Journal of Evolutionary Biology* **23**: 1919–1927.

Savary R, Masclaux FG, Wyss T, Droh G, Cruz Corella J, Machado AP, Morton JB, Sanders IR. 2018. A population genomics approach shows widespread geographical distribution of cryptic genomic forms of the symbiotic fungus Rhizophagus irregularis. *The ISME Journal* **12**: 17–30.

Selosse M-A, Richard F, He X, Simard SW. 2006. Mycorrhizal networks: des liaisons dangereuses? *Trends in Ecology & Evolution* **21**: 621–628.

Selosse M-A, Rousset F. 2011. The plant-fungal marketplace. *Science* **333**: 828–829.

Spatafora JW, Chang Y, Benny GL, Lazarus K, Smith ME, Berbee ML, Bonito G, Corradi N, Grigoriev I, Gryganskyi A, *et al.* 2016. A phylum-level phylogenetic classification of zygomycete fungi based on genome-scale data. *Mycologia* **108**: 1028–1046.

Taudiere A, Munoz F, Lesne A, Monnet A-C, Bellanger J-M, Selosse M-A, Moreau P-A, Richard F. 2015. Beyond ectomycorrhizal bipartite networks: projected networks demonstrate contrasted patterns between early- and late-successional plants in Corsica. *Frontiers in Plant Science* **6**.

Thebault E, Fontaine C. 2010. Stability of ecological communities and the architecture of mutualistic and trophic networks. *Science* **329**: 853–856.

Verbruggen E, Van Der Heijden MGA, Weedon JT, Kowalchuk GA, Röling WFM. 2012. Community assembly, species richness and nestedness of arbuscular mycorrhizal fungi in agricultural soils. *Molecular Ecology* **21**: 2341–2353.

Werner GDA, Cornelissen JHC, Cornwell WK, Soudzilovskaia NA, Kattge J, West SA, Kiers ET. 2018. Symbiont switching and alternative resource acquisition strategies drive mutualism breakdown. *Proceedings of the National Academy of Sciences of the United States of America* **115**: 5229–5234.

Winther JL, Friedman WE. 2008. Arbuscular mycorrhizal associations in Lycopodiaceae. *New Phytologist* **177**: 790–801.

Winther JL, Friedman WE. 2009. Phylogenetic affinity of arbuscular mycorrhizal symbionts in Psilotum nudum. *Journal of Plant Research* **122**: 485–496.

Zanne AE, Tank DC, Cornwell WK, Eastman JM, Smith SA, FitzJohn RG, McGlinn DJ, O'Meara BC, Moles AT, Reich PB, *et al.* 2013. Three keys to the radiation of angiosperms into freezing environments. *Nature* **506**: 89–92.

732 **Tables (1 – 2)**

733

734

735 **Table 1: Effect of the nature of the interaction (i.e. plant carbon nutrition modes)**
736 **on indices of network structure and phylogenetic distributions.**
737 The second column corresponds to *P*-values of Kruskal-Wallis tests for the overall
738 network with, or without (in brackets), the Lycopodiaceae.
739 The three last columns correspond to *P*-values of Whitney U tests (pairwise tests) for
740 the overall network including the Lycopodiaceae.
741 *P*-values less than 5% (significance level) are bolded.

742

743

Index	Kruskal-Wallis test	Whitney U tests		
		AT vs. EMH	AT vs. IMH	IMH vs. EMH
Plant degree (*k*)	**1.1e-19 (1.4e-17)**	**5.3e-16**	**4.2e-7**	0.97
Fungal partner specialization (*Psp*)	**5.6e-11 (1.3e-8)**	**1.1e-4**	**4.5e-9**	0.054
Mean phylogenetic pairwise distance of fungal partners (*MPD*)	**1.2e-4 (2.0e-3)**	**8.0e-4**	**6.8e-3**	0.11

744

745

746

747

748

749

750

751 **Table 2: Fungal sharing between nine entirely (EMH) or initially (IMH)**
752 **mycoheterotrophic plant families.**
753 Number (lower part of the matrix) and percentage (upper part) of fungi shared between
754 family pairs. The last two columns represent *(i)* the total number of fungi shared with
755 other EMH or IMH families, and *(ii)* the number of fungi exclusive to this family (i.e. not
756 shared with any other MH or AT family). The second column indicates the most closely
757 related AT sister-clade of each family; it can be either one family, a higher clade, the

family itself if AT species were compiled in MaarjAM database (e.g. Polygalaceae), or none in the case of Petrosaviaceae (which forms a too divergent distinct branch).

		Most closely related AT sister-clade in our dataset (and divergence time in million years)	Number of plant species	Number of fungal partners	Burmanniaceae	Corsiaceae	Gentianaceae	Petrosaviaceae	Polygalaceae	Triuridaceae	Lycopodiaceae	Ophioglossaceae	Psilotaceae	Total number of shared fungi	Total number of exclusive fungi
EMH	**Burmanniaceae**	Dioscoreaceae **(110 Myr)**	25	38		0%	13%	3%	8%	16%	2%	6%	0%	16	0
	Corsiaceae	Melanthiaceae Liliaceae Smilacaceae **(129 Myr)**	1	1	0		0%	0%	0%	0%	0%	0%	0%	0	0
	Gentianaceae	Apocynaceae **(52 Myr)**	6	8	5	0		0%	9%	8%	0%	0%	0%	6	0
	Petrosaviaceae		1	2	1	0	0		0%	5%	0%	0%	0%	1	0
	Polygalaceae	Polygalaceae **(60 Myr)**	4	4	3	0	1	0		10%	0%	0%	0%	3	1
	Triuridaceae	Dioscoreaceae **(131 Myr)**	4	19	8	0	2	1	2		8%	3%	0%	11	1
IMH	**Lycopodiaceae**	Selaginellaceae **(303 Myr)**	8	7	1	0	0	0	0	2		0%	0%	3	3
	Ophioglossaceae	Aspleniaceae Dryopteridaceae Gleicheniaceae Lygodiaceae Osmundaceae Pteridaceae **(330 Myr)**	5	15	3	0	0	0	0	1	0		6%	4	2
	Psilotaceae		2	2	0	0	0	0	0	0	0	1		1	0

Figure legends (1 – 4)

Figure 1: Framework used in this study to evaluate the constraints upon the emergence of mycoheterotrophic (MH) cheaters in the arbuscular mycorrhizal symbiosis.

(a) Strong phylogenetic constraints (PC) should affect the phylogenetic distributions of MH cheaters and/or their fungal partners; whereas *(b)* functional constraints (FC) should affect the network structure i.e. level of specialization of MH cheaters and/or their partners. Therefore, by investigating specialization and phylogenetic clustering of MH cheaters and of their fungal partners, we evaluated functional and phylogenetic constraints. The patterns and interpretations from the present study are shown in the orange frames.

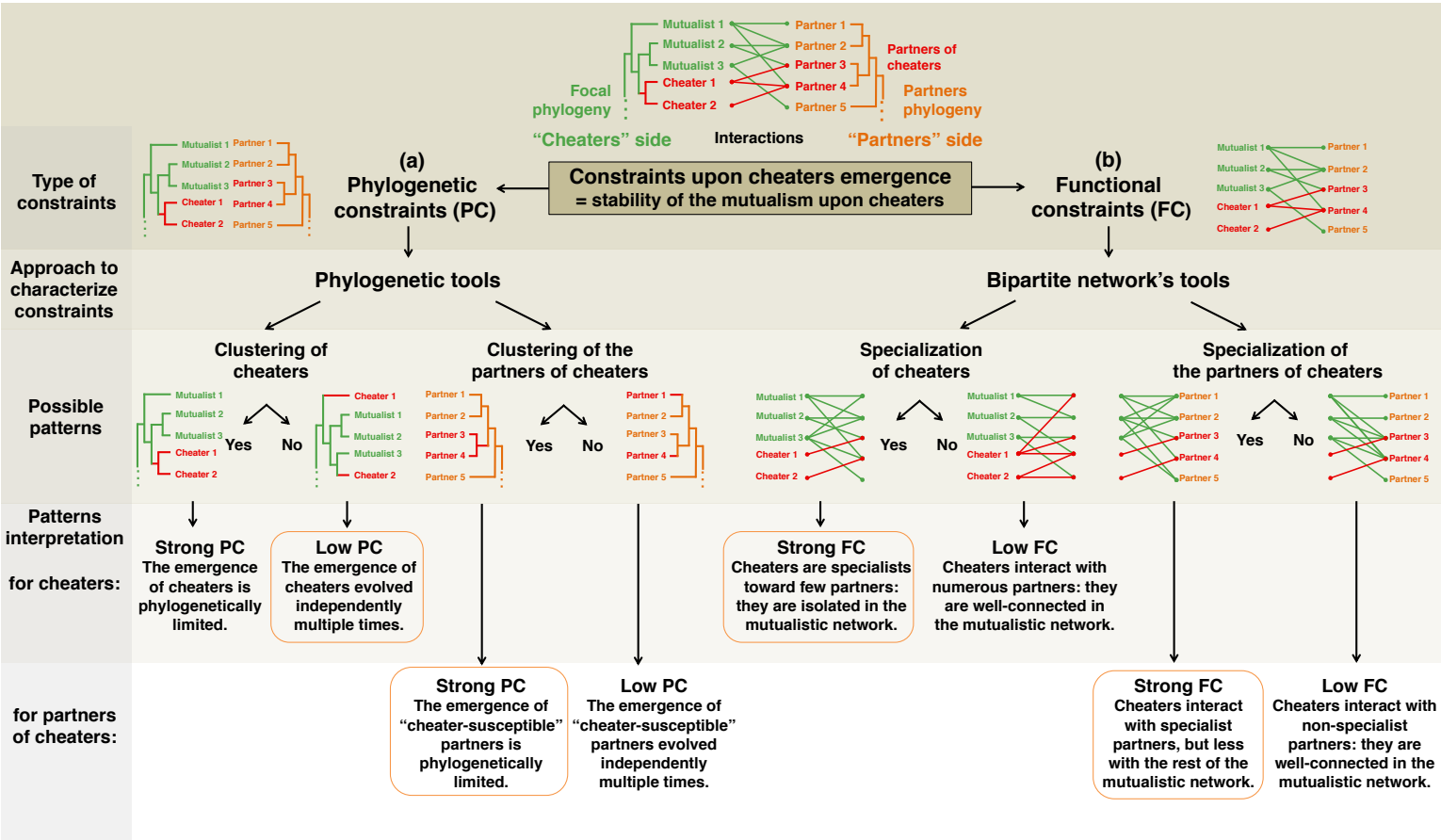
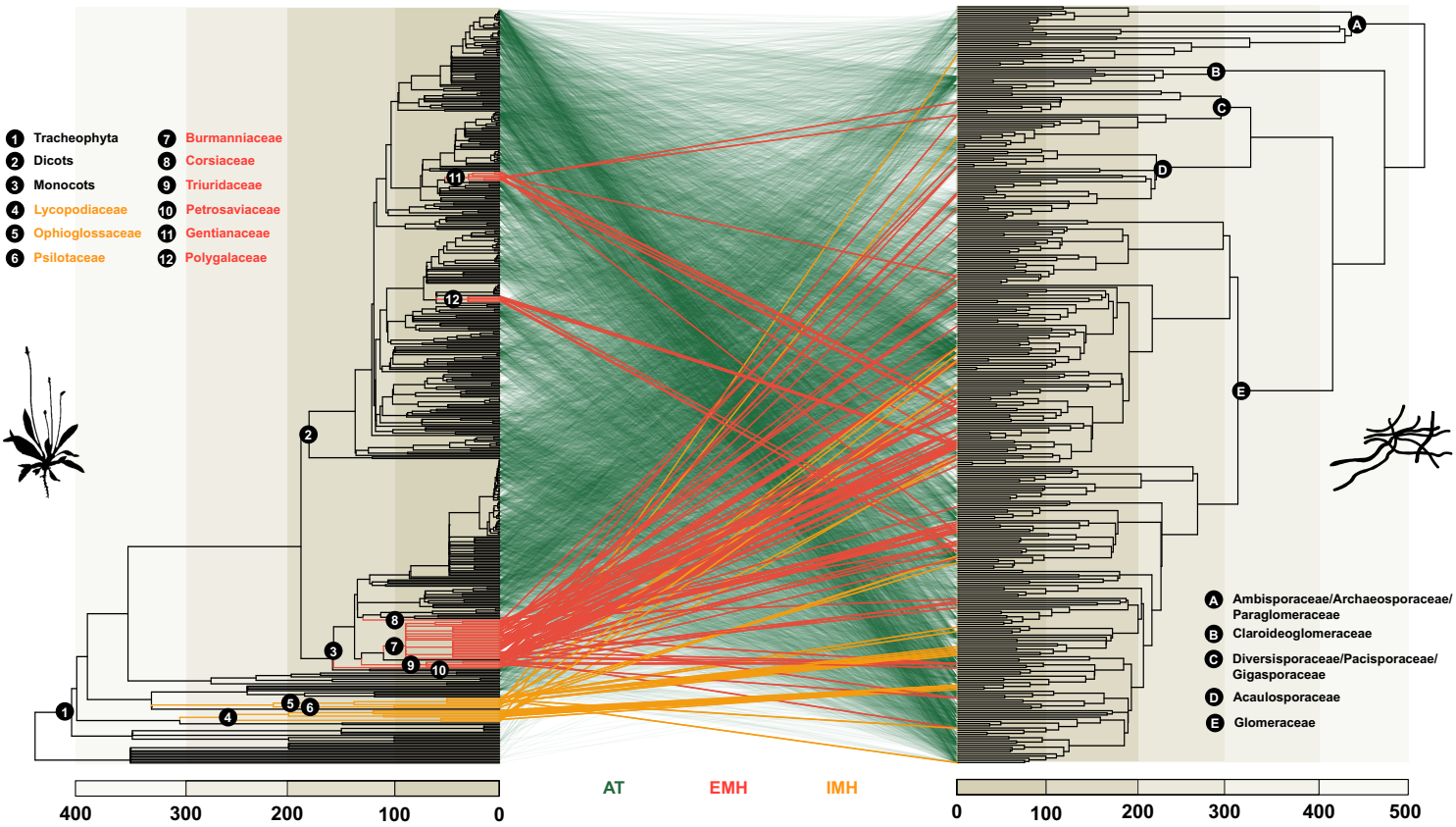
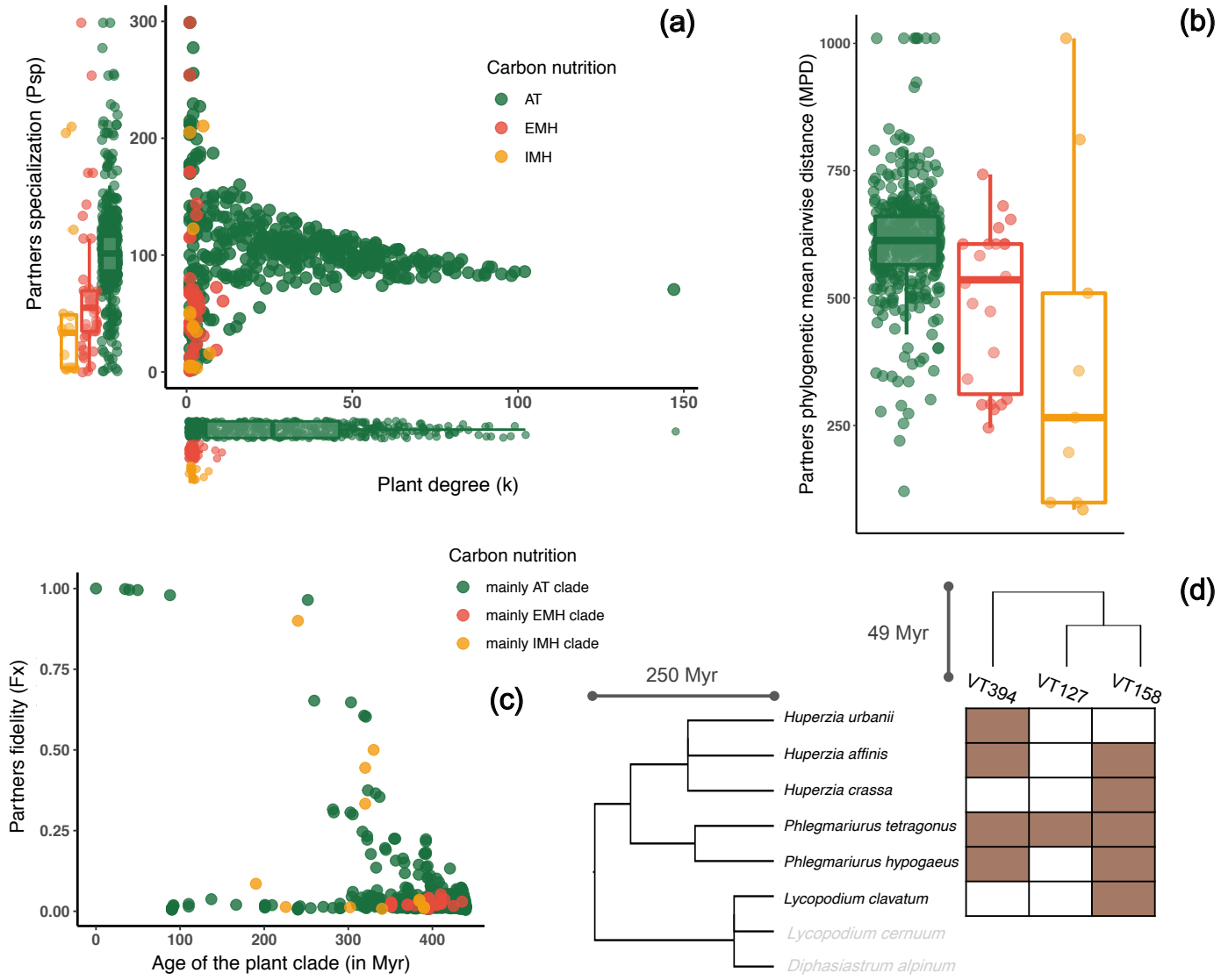


Figure 2: Phylogenetic distribution of mycoheterotroph (MH) cheating in the global arbuscular mycorrhizal mutualism.

Phylogenetic trees of 390 plants (left side) and 351 fungi (right side) forming 26,350 interactions (links) in MaarjAM database. Links are colored according to the autotrophic (green), entirely mycoheterotrophic (red), or initially mycoheterotrophic (orange) nature of the plant. Major plant and fungal clades are named. MH cheaters encompass 41 EMH spp. in 6 families, and 15 IMH spp. in 3 families. Scales of the phylogenetic trees are in million years (Myr).



784 **Figure 3:** Effect of the nature of the interaction on specialization (*k* and *Psp*), partner's
785 mean phylogenetic distance (*MPD*), and partner fidelity (*Fx* - see *List of abbreviations* in
786 Supplementary material):
787 (Categories are defined according to the plant carbon nutrition modes, i.e. AT:
788 autotrophic; EMH: entirely mycoheterotrophic over development; and IMH: initially
789 mycoheterotrophic in the development).
790 **(a):** Plant degree (*k*) against fungal partner specialization (*Psp*) (i.e. the average degree
791 of fungal partners); dots in the bottom left corner indicate reciprocal specialization.
792 **(b):** Mean phylogenetic pairwise distance (*MPD*) of the sets of fungal partners. Boxplots
793 present the median surrounded by the first and third quartile, and whiskers extended to
794 the extreme values but no further than 1.5 of the inter-quartile range.
795 **(c):** Fidelity (*Fx*) toward fungal partners in relation to the age of the plant clade. Clades
796 are defined according to their main carbon nutrition mode of their plants (over 50%).
797 The yellow dots departing from other MH clades (high *Fx* values) correspond to clades
798 of Lycopodiaceae.
799 **(d):** Independent network between of the clubmoss family Lycopodiaceae (rows) and
800 their three arbuscular mycorrhizal fungi (columns) with their respective phylogenetic
801 relationships.
802

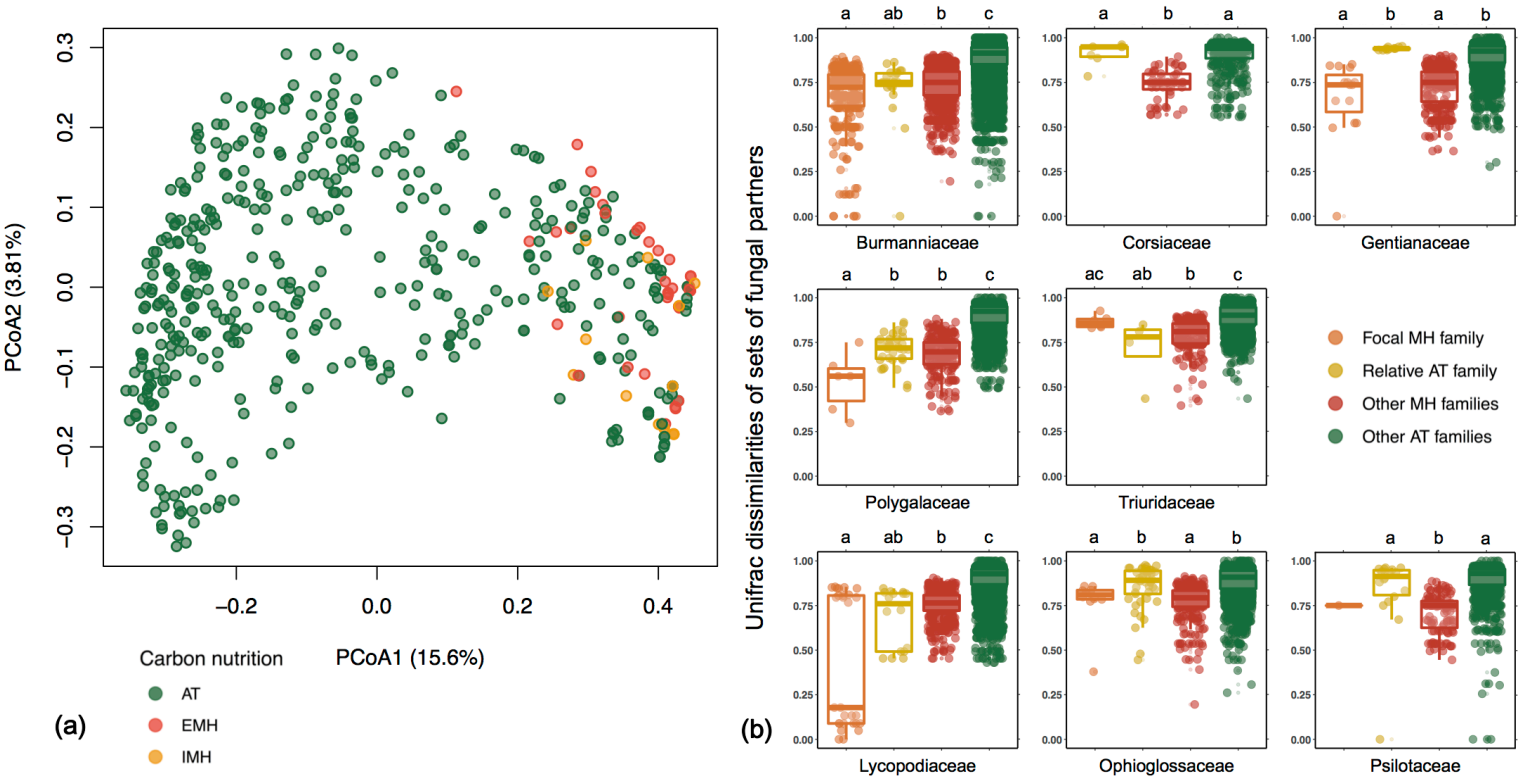

(a)
Partners specialization (Psp)
Plant degree (k)
Carbon nutrition
AT
EMH
IMH
(b)
Partners phylogenetic mean pairwise distance (MPD)
(c)
Partners fidelity (Fx)
Age of the plant clade (in Myr)
Carbon nutrition
mainly AT clade
mainly EMH clade
mainly IMH clade
(d)
250 Myr
49 Myr
VT394
VT127
VT158
Huperzia urbanii
Huperzia affinis
Huperzia crassa
Phlegmariurus tetragonus
Phlegmariurus hypogaeus
Lycopodium clavatum
Lycopodium cernuum
Diphasiastrum alpinum

803

Figure 4: Dissimilarities between sets of fungal partners associated according to the nature of the interaction.

(a): Principal coordinates analysis (PCoA) from UniFrac dissimilarities of sets of fungal partners. Every dot corresponds to a plant species and is colored according to its autotrophic (green), entirely mycoheterotrophic (red), or initially mycoheterotrophic (orange) nature. Only the first two principal axes explaining respectively 15.6% and 3.8% of the variation were kept.

(b): Dissimilarities between sets of fungal partners associated with different MH plant families. For each MH family, Unifrac dissimilarities of sets of fungal partners are calculated between one particular MH species belonging to the focal MH plant family and another plant species (from the same family, from the closest related AT family, from other MH families, or from other AT plant families). All the groups cannot be calculated for every MH family, due to the low number of species within families Corsiaceae and Psilotaceae.



Supporting Information

Supplementary methods:

- Details on the phylogenetic reconstructions
- List of abbreviations

Supplementary table 1: Data selection from MaarjAM: description of the used filters.

Supplementary table 2: Number and percentage of MH plants and their associated fungi.

Supplementary table 3: Independent sub-networks (modules) in the overall arbuscular mycorrhizal network and their respective numbers of partners.

Supplementary table 4: Effect of MH cheating on the network nestedness.

Supplementary Table 5: Null-model to assess the significance of the effect of the nature of the interaction on indices of network structure and phylogenetic distributions.

Supplementary Table 6: Pairwise comparisons of Unifrac dissimilarities between set of fungal partners associated with different plant families.

Supplementary Table 7: Measure of the phylogenetic distributions of MH cheating: measure of NRI (net relatedness index) and NTI (nearest taxon index).

Supplementary Table 8: Effect of the nature of the interaction (i.e. plant carbon nutrition modes) on indices of the network structure in South America and Africa.

Supplementary Figure 1: Rarefaction curves representing the number of fungal taxa as a function of the sampling fraction.

Supplementary Figure 2: Calibrated phylogenetic tree of the 490 plant species.

852 **Supplementary Figure 3:** Calibrated phylogenetic tree of the 351 arbuscular
853 mycorrhizal fungi.
854
855 **Supplementary Figure 4:** Fidelity of plant partners (*Fx*) according to the age of the
856 fungal clade.
857
858 **Supplementary Figure 5:** Effect of the nature of the interaction on specializations in
859 the South American network and the African network.
860
861 **Supplementary figure 6:** The global geographic distribution of sampling sites used in
862 our analysis.